ORIGINAL ARTICLE

Prognostic Role of c-Jun Activation in Patients with Areca Quid Chewing-related Oral Squamous Cell Carcinomas in Taiwan

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Background: Overexpression or activation of c-Jun has been implicated in the pathogenesis of several types of cancer. Treatment of oral cells with areca nut extract in culture can increase *c-Jun* proto-oncogene mRNA levels. The purpose of this study was to investigate the possible role of c-Jun activation in the pathogenesis and prognosis of areca quid chewing-related oral cancer in Taiwan.
Methods: We immunohistochemically examined c-Jun protein activation in human oral squamous cell carcinomas (SCC) using an anti-phosphospecific c-Jun (pc-Jun) antibody on paraffin-embedded sections.
Results: Positive pc-Jun staining was observed in 42 of 70 (60%) cases of oral SCC. No significant correlation was found between pc-Jun expression and patients' age, gender, oral habit, cancer location, clinical stage, tumor size and lymph node status. Kaplan-Meier curves showed that oral SCC patients with positive pc-Jun staining or positive lymph node metastasis had significantly shorter overall survival ($p < 0.018$ and 0.001, respectively, by log-rank test).
Conclusion: These results indicate that c-Jun activation may play an important role in the carcinogenesis of oral SCC. Positive pc-Jun staining may serve as an adjuvant marker of worse prognosis in patients with oral SCC in Taiwan. [*J Formos Med Assoc* 2006;105(3):229–234]

Key Words: c-Jun activation, oral cancer, prognosis

The chewing of areca nut (*Areca catechu*), also called betel nut, preparations has long been associated with the high incidence of oral submucous fibrosis and oral cancer in India and many Southeast Asian countries.[1] In Taiwan, approximately 2 million people have an areca quid (AQ) chewing habit.[2] The ingredients and method used to make AQ in Taiwan differ from that used in other parts of the world; most chewers consume AQ that is made by cutting the fresh areca nut into halves and sandwiching between a piece of *Piper betle* (PB) inflorescence with lime mixture, with or without PB leaf. No tobacco is added to the quid. This chewing habit is also a significant risk factor for malignant transformation from leukoplakia to oral carcinoma after adjustment for other relevant risk factors, whereas the effect of smoking on malignant transformation is not significant.[3]

Recently, we found that treatment of oral mucosal cells with areca nut extract or arecoline in culture induced a threefold increase in *c-Jun* proto-oncogene mRNA levels.[4] The *c-Jun* gene encodes a 39 kDa nuclear protein that is a major component of the mammalian transcription factor activator protein-1 (AP-1) and is involved in cellular proliferation, transformation and apoptosis.[5,6] The activity of c-Jun protein is positively regulated

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by N-terminal phosphorylation at serines 63 and 73 (Ser63/73),[7] and can positively regulate cell proliferation through the repression of tumor suppressor gene *p53* expression and induction of cyclin D1 transcription.[5] In human malignancies, c-Jun overexpression or activation has been found in a wide variety of human tumors, including liver,[8] non-small cell lung,[9] endometrial,[10] head and neck,[11,12] pancreatic,[13] breast[14] and oral cancers.[15] In addition, high levels of c-Jun expression or activation have been associated with poor prognosis in non-small cell lung,[9] breast,[14] endometrial[10] and pancreatic cancers.[13] However, no previous study on the relationship between c-Jun expression or activation and overall patient survival in oral squamous cell carcinomas (SCC) has been reported.

We previously showed that overexpression of cyclin D1 occurred in 83% of oral SCC and was associated with poor prognosis.[16] The transcriptional activation of c-Jun by areca nut extract and overexpression of cyclin D1 in oral SCC led us to postulate that c-Jun may also play a role in the pathogenesis of oral SCC. In this study, we used an immunohistochemical technique to investigate c-Jun activation in oral SCC. The immunostaining results were further correlated with clinicopathologic characteristics of the tumors and patient survival to investigate a possible influence of c-Jun activation on the progression and prognosis of oral SCC in Taiwan.

Methods

Formalin-fixed, paraffin-embedded specimens from 70 patients (63 men, 7 women) with primary oral SCC were included in this study. Diagnosis was made by histologic examination of oral lesions on hematoxylin and eosin-stained slides. All patients received total surgical excision of the cancers at the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital, Taipei, Taiwan, between 1996 and 2002; specimens were obtained from incisional biopsies or total surgical excision of the lesions. If a lymph node was found to be positive for SCC, neck dissection and postoperative radiation therapy were also included in the treatment protocol. None of the patients had received cancer therapy before their initial biopsies. Details of the patients' oral habits, including daily consumption of AQ and cigarettes as well as the duration of these habits, were also recorded. Ten biopsy specimens of normal oral mucosa (NOM) were obtained from 10 non-AQ-chewers during extraction of impacted permanent lower third molars after obtaining informed consent, and used as the controls.

Immunohistochemical staining was performed using a streptavidin-biotin immunoperoxidase technique as previously described.[16] Briefly, sections were deparaffinized and then treated with 3% H_2O_2 to remove endogenous peroxidase activity. After blocking nonspecific binding with 10% normal goat serum (Zymed Laboratories, San Francisco, CA, USA), sections were incubated with mouse monoclonal anti-human phosphospecific c-Jun (Ser63) antibody (pc-Jun; KM-1, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4° C. According to the manufacturer's instructions, this antibody can be used for detection of c-Jun p39 phosphorylated on serine-63 of mouse and human origin by Western blot, immunoprecipitation and immunostaining of formalin-fixed, paraffin-embedded cancer specimens.[17,18] No cross-reaction with Jun-B or Jun-D phosphorylated on the analogous serine residues or with c-Jun non-phosphorylated at serine-63 was detected. Bound antibodies were detected by sequential incubations with biotinylated goat anti-mouse IgG antibody, streptavidin-peroxidase conjugate and diaminobenzidine (Zymed). The sections were then counterstained with hematoxylin and examined by light microscopy. The primary antibody was replaced by non-immune mouse serum as a negative control.

Specimens were examined under a light microscope and epithelial nuclear staining was counted. The grade of pc-Jun expression in each specimen was evaluated according to the percentage of positively stained cells among the total number of counted cancer or epithelial cells. Although there

was variability in the total number of cells counted in each specimen, the percentage of positively stained cells was estimated by counting 300 cells per area from five varied areas and represented as labeling indices (LI). The tumor specimens were considered positive for pc-Jun expression when more than 10% of tumor cells were stained. The correlation between pc-Jun expression and clinicopathologic parameters of oral SCC was analyzed by Fisher's exact test using SAS version 8.0 (SAS Institute Inc, Cary, NC, USA). Cumulative survival was analyzed with the Kaplan-Meier product-limit method. The duration of survival was measured from the beginning of treatment to the time of death or the last follow-up. Comparison of cumulative survival between groups was performed using the log-rank test with the Statistica program (StatSoft Inc, Tulsa, OK, USA).

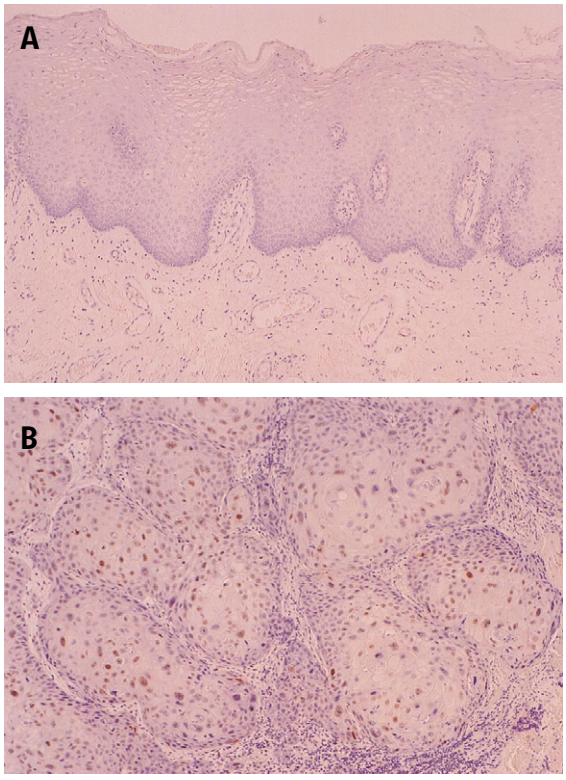

Figure 1. Typical staining patterns for pc-Jun: (A) normal buccal mucosa showing negative pc-Jun staining in all epithelial cells (× 100); (B) well-differentiated oral squamous cell carcinoma showing positive pc-Jun nuclear staining in about 80% of cancer cells (× 100).

Results

To investigate the impact of c-Jun activation in oral SCC, immunohistochemical analysis was performed using an anti-pc-Jun antibody on paraffin-embedded sections. Typical staining patterns for pc-Jun are shown in Figure 1. In oral SCC, positive pc-Jun staining was mainly nuclear and found evenly in tumor epithelial cells (Figure 1A) and occasionally in associated stromal cells. In NOM, most cases showed negative pc-Jun staining (Figure 1B), whereas positive staining was detected in a few basal epithelial cells (mean LI = 5.5 ± 1.5%) in four cases. Therefore, we used 10% (approximately mean ± 2 SD) as the cut-off value to differentiate positive versus negative c-Jun activation for subsequent analyses, and found c-Jun activation in 42 of 70 (60%) cases of oral SCC.

The relationships between pc-Jun LI in specimens of oral SCC and clinicopathologic parameters of 70 oral SCC patients are shown in the Table. No significant correlation was found between pc-Jun LI and age, gender, cancer location, clinical staging, primary tumor TNM status, AQ chewing or smoking habits, or histologic differentiation of SCC at the time of initial presentation ($p > 0.05$). Kaplan-Meier analysis showed that patients with tumors containing > 10% pc-Jun-positive cells had significantly shorter overall survival than those with tumors containing ≤ 10% ($p < 0.018$, Figure 2A). Patients with positive lymph node status also had significantly shorter overall survival ($p < 0.001$, Figure 2B).

Discussion

de Sousa et al used non-phosphorylated c-Jun polyclonal antibody to investigate c-Jun expression in NOM and oral SCC, and found that c-Jun was detected in the cytoplasm of suprabasal cells in NOM, while in oral SCC, c-Jun was detected in all the nuclei of the cancer cells.[15] It has been reported that phosphorylation of c-Jun is the key event in the control of AP-1 activity.[7] Therefore, we used a phosphospecific c-Jun antibody to investigate c-Jun activation in oral SCC, which was positive in 60% of cases. These results indicate that there is a pool of total c-Jun in oral SCC, and

Table. Correlation between pc-Jun expression in oral squamous cell carcinoma (SCC) and the clinicopathologic parameters of 70 oral SCC patients

	Degree of c-Jun staining			*p*
	0–10%	11–50%	≥ 51%	
Age (yr)				0.866
< 40	2	2	3	
40–49	8	3	6	
50–59	9	7	4	
60–69	8	8	6	
≥ 70	1	2	1	
Gender				0.787
Male	25	19	19	
Female	3	3	1	
Cancer location				0.379
Buccal mucosa	10	9	12	
Gingiva	5	1	2	
Tongue	8	6	4	
Palate	1	4	0	
Oropharynx	4	2	2	
Clinical stage				0.243
1	4	4	6	
2	12	6	2	
3	4	4	2	
4	8	8	10	
T status				0.516
T1	5	5	9	
T2	14	11	7	
T3	2	1	0	
T4	7	5	4	
Lymph node involvement				0.852
Yes (N1 + N2 + N3)	8	8	7	
No (N0)	20	14	13	
Oral habit				0.512
AQ chewer and smoker	21	15	14	
AQ chewer only	1	0	0	
Smoker only	1	3	4	
Non-AQ chewer and nonsmoker	5	4	2	

AQ = areca quid.

that a substantial proportion of this protein is in an active, serine 63-phosphorylated state.

Extracellular signals including growth factors (e.g. epidermal growth factor and transforming growth factor-α), transforming oncoproteins (e.g. Ha-ras, raf-1, c-sis), cytokines and UV have been shown to stimulate c-Jun phosphorylation at Ser63/73 and activate c-Jun-dependent transcription.[7] In the case of cell transformation, overexpression of c-Jun can lead to malignant transformation of immortalized rat fibroblasts while transformation of rat embryo fibroblasts by c-Jun requires cooperation with activated ras.[19] This oncogenic cooperation is partially dependent on c-Jun phosphorylation at Ser63/73.[20] Young et al showed that transgenic mice expressing c-Jun dominant-negative mutant (TAM67) are resistant to DMBA-TPA-induced skin papillomagenesis without evidence of skin anomalies.[21] Furthermore, skin tumor development caused by constitutive activation of the ras pathway was impaired in Ser63/73 mutant mice, supporting the significance of c-Jun phosphorylation in ras-induced carcinogenesis.[22] We previously showed that mutations and overexpression of *ras* oncogenes are important in the oncogenesis of AQ chewing-related human oral SCC in Taiwan.[23,24] Therefore, the co-activation of c-Jun and ras may play an important role in the pathogenesis of oral SCC in Taiwan.

It has been shown that c-Jun overexpression increased expression of a matrix-degrading enzyme MMP-9 and *in vitro* chemoinvasion in MCF7 breast cancer cells.[25] Furthermore, stable expression of TAM67 mutant can effectively inhibit 12-O-tetradecanoylphorbol-13-acetate-induced invasion in mouse keratinocytes.[26] A relationship between c-Jun expression and an invasive/metastatic potential has also been noted for several human tumors. Yokoyama et al showed that c-Jun expression was related to lymph node metastasis in endometrial carcinomas.[10] Volm et al found that c-Jun overexpression in primary lung carcinomas was significantly correlated to lymph node involvement.[27] Tiniakos et al showed that the intensity of c-Jun immunostaining was significantly related to tumor stage in transitional cell carcinomas (TCC) of the urinary bladder and found a positive association between an intense c-Jun immunoreactivity and muscle invasive growth.[28] Furthermore, Gee et al found that increased c-Jun activation in human breast cancer was significantly associated with distant metastasis.[14] In contrast, Nephew et al showed that expression of c-Jun does not cor-

relate with endometrial cancer stage and grade.[29] In the present study, no significant correlation was found between the LI of pc-Jun and patients' STNM status. In addition, the LI of pc-Jun was found at a similar level in both early and advanced stages of oral SCC. Thus, c-Jun activation may be an early event in oral carcinogenesis.

It has been shown that patients with non-small cell lung,[9] breast,[14] endometrial[10] and pancreatic cancers[13] exhibiting c-Jun overexpression or activation have a poor prognosis. However, some studies found no significant correlation between c-Jun expression and overall survival in patients with hepatocellular carcinoma,[8] non-small cell lung cancer,[27] or TCC of the urinary bladder.[28] In head and neck SCC, Riva et al showed that overexpression of c-Jun is associated with the absence of tumor response to neoadjuvant chemotherapy or radiotherapy and with tumor relapse, but fails to predict patients' long-term survival.[11] Miura et al assessed c-Jun expression in head and neck SCC after radiation therapy and found that c-Jun expression in residual or relapse tumors was higher than in tumors showing complete regression.[12] This study showed that patients with tumors containing > 10% pc-Jun-positive cells had significantly shorter overall survival. Thus, pc-Jun positive staining may serve as an adjuvant marker of poor survival in patients with oral SCC in Taiwan.

In conclusion, this study demonstrated a high incidence of c-Jun activation in oral SCC in Taiwan. Furthermore, c-Jun activation correlated with poor overall survival. To our knowledge, this is the first study to show that c-Jun activation correlates with overall survival in patients with oral SCC. We suggest that c-Jun activation may be included with other prognostic factors used to characterize subsets of oral SCC patients with a poor outcome, and that c-Jun antagonists such as curcumin[30] may have a role in the chemoprevention and therapy of oral cancer.

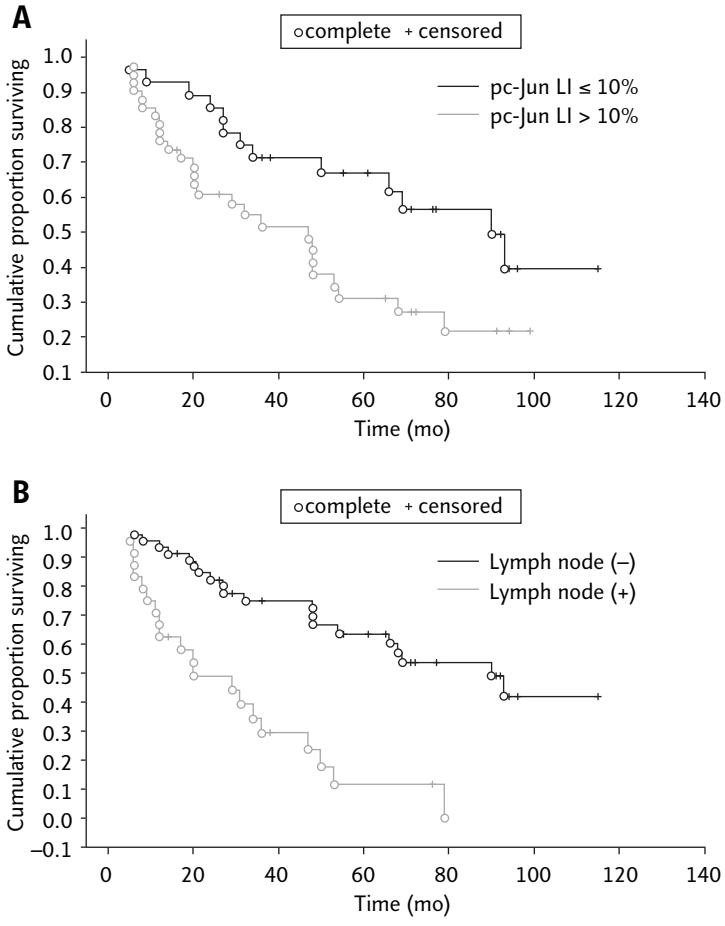

Figure 2. Kaplan-Meier survival curves of 70 patients with oral squamous cell carcinoma. (A) Patients with tumors containing > 10% pc-Jun-positive cells had significantly shorter overall survival than those with tumors containing ≤ 10% pc-Jun-positive cells ($p < 0.018$). (B) Patients with positive lymph node metastasis had significantly shorter overall survival ($p < 0.001$). The duration of survival was measured from the beginning of treatment to the time of death (complete) or the last follow-up (censored). LI = labeling indices.

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